

The Effect of Nitric Oxide Synthases Inhibitors on Inflammatory Bowel Disease in a Rat Model

CHARALAMBOS J. PILICHOS¹, ILIAS A. KOUERINIS², GEORGE C. ZOGRAFOS²
DIMIRIS P. KORKOLIS³, ATHENA A. PREZA¹, MARIA GAZOULI³, EVANGELOS I. MENENAKOS²,
ANTONIOS E. LOUTSIDIS³, FLORA ZAGOURI², VASSILIS G. GORGOULIS³ and CONSTANTIN I. FOTIADIS¹

¹Third Department of Propeudeutic Surgery, University of Athens, Sotiria Hospital, Athens;

²First Department of Propeudeutic Surgery, University of Athens, Hippokration Hospital, Athens;

³Department of Histology-Embryology (Molecular Carcinogenesis Group),
School of Medicine, University of Athens, Greece

Abstract. *Background/Aims:* Overexpression of nitric oxide (NO) has been implicated in the pathogenesis of experimental and clinical inflammatory bowel disease (IBD). NO is produced by two types of enzymes: constitutively expressed and inducible NO synthases (NOS). This study assessed N^w-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AMG), the most studied inhibitors of nitric oxide synthases, with regard to their effectiveness as modulators of inflammation in trinitrobenzene sulfonic acid (TNBS)-induced colitis in the rat. *Materials and Methods:* Colitis was induced in Wistar rats. The colitis was treated everyday for 10 days with L-NAME and AMG. To assess the severity of the colitis, clinical (body weight), hematological (hematocrit and erythrocytes sedimentation rate-ESR) and morphological (gross and microscopic) criteria were used. *Results:* The administration of both nitric oxide synthases inhibitors L-NAME and AMG proved to be beneficial in all the examined parameters compared with the control group. A statistically significant difference between the L-NAME and the AMG groups was observed only in macroscopic and histological grading. *Conclusion:* NOS inhibitors may be promising agents in preventing the onset, or mediating the symptoms, of inflammatory bowel disease.

Over the last decade the pathophysiological role of nitric oxide (NO) in the pathogenesis of inflammatory bowel disease (IBD) has been well documented in both patients (1) and animal models with experimentally-induced colitis (2). Nitric oxide is synthesized by various cell types, via the

so-called "L-arginine-NO pathway" (3). The main catalyzing enzyme in this metabolic pathway (nitric oxide synthase-NOS) is present, in the gut, in two isoforms: the first, Ca²⁺-dependent isoform, is called constant NOS (c-NOS) and catalyses the production of minimal amounts of NO which are necessary for maintaining the physiological gut functions (motility, absorption and secretion); the second isoform is called inducible-NOS (i-NOS), is Ca²⁺-independent and, once stimulated, synthesizes excessive amounts of NO, leading to severe intestinal injury. This is considered to be a mechanism of major importance in the pathogenesis of colonic inflammation (4-6).

It is well established that active ulcerative colitis in humans is associated with increased cytokine production (7). This induces the i-NOS synthase in a variety of inflammatory cells including macrophages and neutrophils, as well as in vascular endothelium and smooth muscle (8), which are responsible for an inflammatory response.

The aim of this study was to compare, in a rat model of experimental colitis, the effect of the two most studied NOS inhibitors, N^w-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine (AMG), which present a different selectivity for each of the NOS isoforms (AMG being more selective for the i-NOS than L-NAME), and investigate their possible application in patients with IBD by reviewing the literature.

Materials and Methods

Animals and experimental groups. Seventy-five male Wistar rats (Institute Pasteur, Athens, Greece), weighing 300-450 g, were used in all experiments. The animals were maintained in 48-hour dark/light cycles and had free access to food and water based on the Guide for the Care and Use of Laboratory Animals, NRC (9). According to the treatment, the rats were randomized into three experimental groups: Group A (n=25): Colitis + no treatment; Group B (n=25): Colitis + L-NAME; Group C (n=25): Colitis + AMG.

Correspondence to: Dr. V.G. Gorgoulis, 53 Antaiou Str., Ano Patisia, Athens, Greece, 111 46. Fax: +302106535894, e-mail: histoclub@ath.forthnet.gr

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Table Ia. *Gross anatomical scoring system (11).*

Macroscopic criteria		
Lesion	Score	
Adhesions	None	0
	Minimal	1
	Involving several bowel loops	2
Strictures	None	0
	Mild	2
	Severe	3
Ulcers/Scars	None	0
	One linear ulcer/scar<1cm	1
	Multiple linear ulcers/scars<1cm	2
	One large ulcer/scar<1cm	3
Wall thickness	<1mm	0
	1-3mm	1
	>3mm	2
Hyperemia	Absent	0
	Present	1
Mesentery bleeding	Absent	0
	Present	1
Maximum score	12	

Table Ib. *Histological grading system (4).*

Histological score		
Lesion	Score	
Loss of normal mucosal architecture	None	0
	Mild	1
	Moderate	2
Cellular infiltration	Severe	3
	Normal	0
	Mild	1
Muscle thickening	Moderate	2
	Transmural	3
	None	0
Crypt abscesses	Mild	1
	Moderate	2
	Severe	3
Goblet cell depletion	Absent	0
	Present	1
Maximum score	11	

The observation period was set at ten days, after the induction of colitis.

Induction of colitis and drug administration. Following standard operating procedure, colitis was induced under general anesthesia with ketamine (0.3 ml) and midazolam (0.3 ml), by intrarectal instillation of 0.1 ml trinitrobenzene sulphonic acid -TNBS (60mg/ml in 30% ethanol) through a plastic catheter approximately 3 cm proximal to the anus (10).

Table II. *Obtained results.*

	Controls	L-name	AMG	<i>p</i>
Decrease of b.w. (gr)	29.8±18.5	19.6±17.3*	11.2±13.9*	0.03
Hematocrit (%)	51.6±9.6	64.5±9.3*	61.2±10.1*	0.12
WBC (10 ³ /μL)	2548.5±1293	1831.4±894*	1933±952*	0.34
Platelets (10 ³ /μL)	1158±473	972±279*	943±195*	0.33
ESR (mm)	8.1±3.5	3.6±2.5*	4.3±3*	0.18
Macroscopic score	8.4±2.7	5.4±2.8*	3.8±3.2*	0.03
Histological score	8.9±2.3	6±2.8*	4.1±3*	0.01

The results in all experimental groups are expressed as mean ± standard deviation. (*p* indicates the *p*- value for differences observed between the L-NAME and AMG groups, while * represents *p*<0.05 compared with the control group).

Both NOS inhibitors, L-NAME (15 μmol/kg/day) and AMG (15 μmol/kg/day), were administered to rats *per os*, beginning one day before the induction of colitis and continuing for the entire 10-day observation period. The above-mentioned concentrations of L-NAME and AMG have been well demonstrated to produce near maximal inhibition of c-NOS and i-NOS, respectively (5).

Assessment of the severity of colitis. All rats were sacrificed 10 days after the induction of colitis, by an overdose of anesthetic agents. Blood samples were taken directly from the left ventricle and the entire colon was excised and opened longitudinally. After gross examination, samples of colonic tissue were fixed in neutral buffered-formalin and processed for subsequent histological examination. To assess the severity of colitis, clinical (body weight), hematological (hematocrit and erythrocytes sedimentation rate-ESR) and morphological (gross and microscopic) criteria were used. The observed lesions were graded by observers unaware of the therapeutic protocols, using a minor modification of the Vilaseca (11) and McCafferty (4) scoring system (Table I).

Statistical analysis. Statistical analysis of the obtained results was performed by the Student's *t*-test and the statistical significance level was set at *p*<0.005.

Results

Table II and Figure 1 summarize the obtained results based on decrease of body weight, the hematological parameters and the anatomic scores in all experimental groups. The administration of both nitric oxide inhibitors L-NAME and AMG seemed to be beneficial in all the above-mentioned parameters compared with the control group. Furthermore, a statistically significant difference between the L-NAME and the AMG group was noticed only in macroscopic and

histological grading. Of particular interest was the fact that normal histology (Score=0) was exclusively observed in 5/25 animals (20%) in the AMG group.

Discussion

The aim of this study was to estimate the therapeutic efficacy of L-NAME and AMG, in an experimental model of IBD in rats. Among various animal models we chose the TNBS-induced colitis because of its simplicity, reproducibility, low cost and the good documentation available in the literature (12).

Numerous pharmacological properties of these new drugs have been investigated in detail, but undoubtedly some aspects must be further elucidated. Although, the selectivity of L-NAME and AMG for c-NOS and i-NOS, respectively, is adequately documented (13, 14), it should be noted that neither of the molecules is absolutely selective for either isoenzyme (5). Furthermore, the pathophysiological role of NO and NOS in the gut presents major controversies. Nitric oxide is a molecule with a paradoxical physiological action in the gut, being labeled with epithets such as "Jekyll and Hyde" (15). Nitric oxide plays a dual role in modulating the inflammatory response, by virtue of its ability to act as both an anti-inflammatory and a proinflammatory agent. The current aspect suggests that NO produced by the constitutive isoform of NOS in small amounts is beneficial, while on the contrary, when it is produced by the inducible isoform of NOS in large quantities, it might be detrimental. According to the above, the selective inhibition of these enzymatic isoforms should be expected to result in a contrary effect. However, such activity was not observed in our model. Both NOS inhibitors attenuated the colonic inflammation and, in terms of clinical features, both molecules proved to be almost equivalent. Although our results are in accordance with the main body of the relative literature (15, 16), different opinions have also been reported. These opinions range from no protection (4) to exacerbation of the intestinal injury (17), while Miller *et al.* have demonstrated that chronic NOS inhibition, in the absence of TNBS, causes significant intestinal inflammation (18). However, if such a beneficial result were expected for AMG, it would seem paradoxical for L-NAME. Why did L-NAME not prove to be detrimental? Kubes (6) presumed that c-NOS may lose its gut protective function and also contribute to the injury process and therefore suggested that L-NAME may present a broader spectrum of anti-inflammatory properties. On the other hand, AMG achieved, within the observation period, a significantly better restitution of the colonic damage compared to L-NAME. This latter may either be due to its selectivity for I-NOS inhibition or due to its direct proliferative effect on the colonic mucosal cells (16). Furthermore, although the side-effects of the two drugs were not studied in our

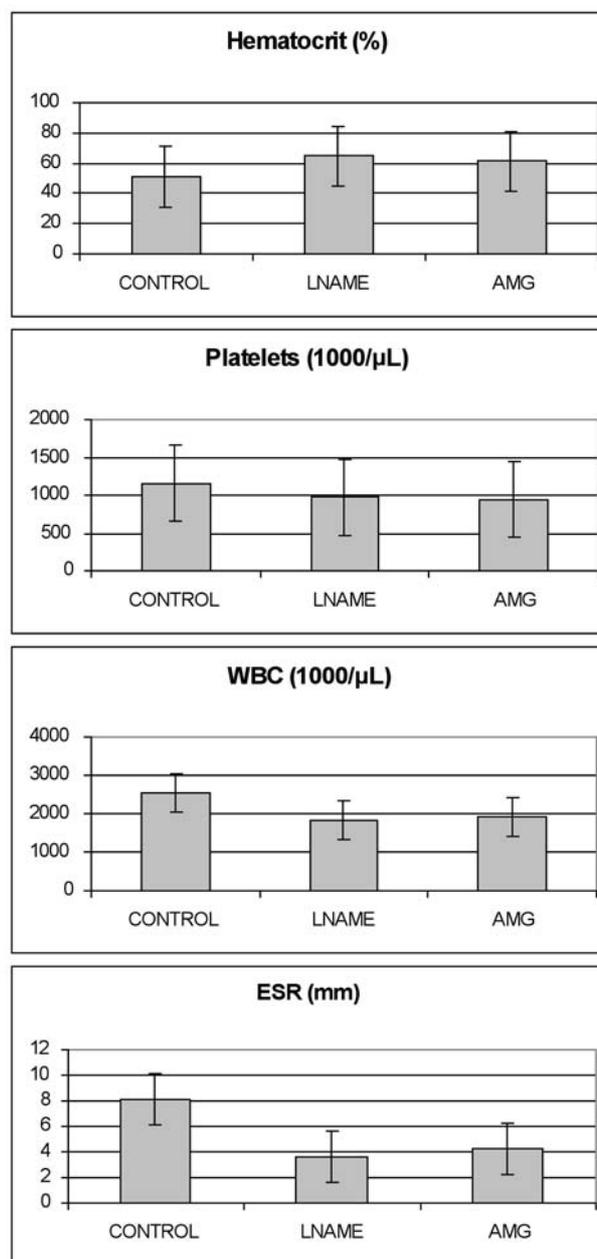


Figure 1. Mean values of hematocrit, platelet number, leukocytes and erythrocyte sedimentation rate.

protocol, it is evidenced that AMG lacks some of the undesirable side-effects of L-NAME, such as severe hypertension and its sequellae, when it enters the systemic circulation (19).

In patients suffering from ulcerative colitis, it is proven that NO synthase activity in colonic mucosa is about 0.55 nmol/min per g tissue; a value that is eight-fold higher than the value observed in healthy individuals (1). This finding

indicates that active ulcerative colitis is associated with a substantial increase of Ca²⁺ -independent NO synthase activity in the colonic mucosa, characteristic of the inducible form of the enzyme. Furthermore, the induction of NO synthase in the mucosa occurs despite corticosteroid therapy, a feature that may reflect some kind of resistance to corticosteroids and, therefore, failure of such therapy to control the inflammatory response.

In conclusion, NOS inhibitors might prove to be promising therapeutic agents for IBD. However, before their clinical implementation in humans, numerous questions concerning their pharmacological properties must be answered. The route, the dose and the proper timing of their administration, in association with a deeper understanding of the pathophysiological role of NO, must be investigated.

References

- 1 Boughton-Smith NK, Evans SM, Hawkey CJ, Cole AT, Balsitis M, Whittle BJ and Moncada S: Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet* 342: 338-340, 1993.
- 2 Southey A, Tanaka S, Murakami T, Miyoshi H, Ishizuka T, Sugiura M, Kawashima K and Sugita T: Pathophysiological role of nitric oxide in rat experimental colitis. *Int J Immunopharmacol* 19: 669-676, 1997.
- 3 Moncada S and Higgs A: The L-arginine-nitric oxide pathway. *NEJM* 329: 2002-2012, 1993.
- 4 McCafferty DM, Miamparriba M, Sihota E, Sharkey KA and Kubes P: Role of inducible nitric oxide synthase in TNBS induced colitis in mice. *Gut* 45: 864-873, 1999.
- 5 Grisham MB, Specian RD and Zimmerman TE: Effects of NOS inhibition on the pathophysiology observed in a model of chronic granulomatous colitis. *JPET* 271: 1114-1121, 1994.
- 6 Kubes P: Inducible nitric oxide synthase: a little bit of good in all of us. *Gut* 47: 6-9, 2000.
- 7 Mahida YR, Wu K and Jewell DP: Enhanced production of interleukin-1 by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut* 341: 465-66, 1989.
- 8 Moncada S, Palmer RMJ and Higgs EA: Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43: 109-42, 1991.
- 9 Guide for the Care and Use of Laboratory Animals. NRC. National Academy Press, p11, 1996.
- 10 Chin KW and Barrett KE: Mast cells are not essential to inflammation in murine model of colitis. *Dig Dis Sci* 39: 513-525, 1994.
- 11 Vilaseca J, Salas A, Guarner F, Rodriguez R, Martinez M and Malagelada JR: Dietary fish oil reduces progression of chronic inflammatory lesions, in a rat model of granulomatous colitis. *Gut* 31: 539-544, 1990.
- 12 Elson CO, Sartor RB, Tennyson GS and Riddell RH: Experimental models of inflammatory bowel disease. *Gastroenterology* 109: 1344-1367, 1995.
- 13 Rees DD, Palmer RM, Schulz R, Hodson HF and Moncada S: Characterization of three inhibitors of endothelial NOS *in vitro* and *in vivo*. *BR J Pharmacol* 101: 746-752, 1990.
- 14 Corbett JA, Tilton RG, Chang K, Hasan KS, Ido Y, Wang JL, Sweetland MA, Lancaster JR Jr, Williamson JR and McDaniel ML: Aminoguanidine a novel inhibitor of nitric oxide formation prevents diabetic vascular formation. *Diabetes* 41: 552-556, 1992.
- 15 Miller MJ, Chotainaruamol S, Sadowska-Krowicka H, Kakkis JL, Munshi UK, Zhang XJ and Clark DA: Nitric oxide: the Jekyll and Hyde of gut inflammation. *Am J Physiol* 39: C180-182, 1993.
- 16 Rachmiliewicz D, Karmeli F, Okon E, Rubenstein I and Better OS: Experimental colitis is ameliorated by inhibition on NOS activity. *Gut* 37: 247-255, 1995.
- 17 Pfeiffer CJ and Qiu BS: Effects of chronic NOS inhibition on TNB-induced colitis in rats. *J Pharm Pharmacol* 47: 827-832, 1995.
- 18 Miller MJ, Munshi UK, Sadowska-Krowicka H, Kakkis JL, Zhang XJ, Eloby-Childress S and Clark DA: Inhibition of calcium dependent NOS causes ileitis and leukocytosis in guinea pigs. *Dig Dis Sci* 39: 1185-1192, 1994.
- 19 Baylis C, Mitruka B and Deng A: Chronic blockade of NO synthesis in the rat produces systemic hypertension and glomerular damage. *J Pharm Pharmacol* 47: 827-832, 1995.

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